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(Article begins on next page)

1 *Escherichia coli* overexpressing a Baeyer-Villiger monooxygenase from *Acinetobacter radioresistens*
2 become resistant to Imipenem

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10 *Running title: *Baeyer-Villiger monooxygenase and antibiotic resistance*

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18 **Keywords:** Antibiotic resistance; BVMO; flavoprotein; *Escherichia coli*; protein turnover; phylogenetics;
19 LC-MS

20

21

22 **ABSTRACT**

23 Antimicrobial resistance is a global issue currently resulting in fatalities of hundreds of thousands a year
24 worldwide. Data present in literature illustrate the emergence of many bacterial species that display
25 resistance to known antibiotics; *Acinetobacter* spp. is a good example of this. We report here that
26 *Acinetobacter radioresistens* has a Baeyer-Villiger monooxygenase (Ar-BVMO) with 100% amino acid
27 sequence identity to Ethionamide monooxygenase of the MultiDrug-Resistant (MDR) *Acinetobacter*
28 *baumannii*. Both enzymes are phylogenetically only distantly related to other canonical bacterial BVMO
29 proteins. Ar-BVMO is not only capable of oxidizing two anticancer drugs metabolized by human FMO3,
30 Danusertib and Tozasertib, but can also oxidize other synthetic drugs such as Imipenem. The latter is a
31 member of carbapenems, a clinically important antibiotic family used in the treatment of MDR bacterial
32 infections. Susceptibility tests performed by the Kirby-Bauer disk diffusion method demonstrate that
33 Imipenem sensitive *E. coli* BL21 cells overexpressing Ar-BVMO become resistant to this antibiotic. Agar
34 disc diffusion assay proves that when Imipenem reacts with Ar-BVMO, it loses its antibiotic property.
35 Moreover, NADPH consumption assay with the purified Ar-BVMO demonstrates that this antibiotic is
36 indeed a substrate and its product is identified by LC-MS as a BV oxidation product of the carbonyl
37 moiety of the β -lactam ring. This is the first report of an antibiotic-inactivating BVMO enzyme that
38 whilst mediating its usual BV oxidation, is also operating by an unprecedented mechanism of carbapenem
39 resistance.

40

41 INTRODUCTION

42 Bacteria classified within the genus *Acinetobacter* play an increasingly important role in
43 pathogenesis of human diseases. Among members of this genus, *Acinetobacter baumannii* is the most
44 frequently isolated species from humans (found in endotracheal aspirate, blood, peri-anal and wound
45 secrets) where it is known to manifest multidrug-resistance (MDR) (1). *Acinetobacter* species are widely
46 distributed in nature and can be found in soil, water, sewage, and in variety of foodstuffs (1-2). They are
47 present in the hospital environment especially intensive care units (3-4) as they are inhabitants of healthy
48 human skin as part of normal flora and can be isolated from dry surfaces and equipment and, they can
49 easily survive for many days or weeks, even in dry conditions (1-2). Increasing recovery of the MDR *A.*
50 *baumannii* in nosocomial settings is a frightening reality (5,6) and the combination of its environmental
51 resilience and its wide range of resistance determinants renders it a successful nosocomial pathogen (6).
52 These MDR strains often spread to cause outbreaks throughout entire cities, countries, and continents (7-
53 10). The importation of MDR strains from areas with high rates of antimicrobial resistance to areas with
54 historically low rate has been demonstrated. United Kingdom and U.S. military and non-military
55 personnel returning from operations in Iraq and Afghanistan harboured infections caused by multi-
56 resistant *A. baumannii* (12-14). Resistance of *A. baumannii* to the carbapenem Imipenem, which is the
57 drug of choice for treating serious infections caused by this species, leads to difficult-to-treat nosocomial
58 infections (7,15-16). Carbapenem resistance results mostly from the expression of acquired carbapenem-
59 hydrolyzing oxacillinases in *A. baumannii*. The blaOXA-23 gene that confers drug resistance in *A.*
60 *baumannii*, codes for the oxacillinase OXA-23, one of the five Ambler class D β -lactamases (17).
61 Outbreaks of OXA-23 producing *A. baumannii* have been reported worldwide and may represent an
62 emerging threat (18). Poirel and co-workers found that *Acinetobacter radioresistens*, a commensal
63 bacterial species that is identified on the skin of hospitalized and healthy patients is the source of the
64 blaOXA-23 gene (19).

65 Recently we have identified a novel Baeyer-Villiger monooxygenase (Ar-BVMO) in *A.*

66 *radioresistens* strain S13 (20). Baeyer-Villiger monooxygenases (BVMOs) are flavin-containing enzymes
67 that mediate specific Baeyer-Villiger oxidations on the carbonyl moiety of substrates. Ketones are
68 converted into the corresponding esters and lactones by the Baeyer-Villiger reaction. BVMO enzymes are
69 abundant in bacterial, fungal and plant genomes but they are absent in animal and human genomes. All
70 characterized BVMOs contain a flavin cofactor that is crucial for catalysis while NADH or NADPH is
71 required as electron donor. Most reported BVMOs are soluble proteins in contrast to many other
72 monooxygenase systems that are often found to be membrane bound. BVMOs are divided into three
73 different types according to their general characteristics (21). Type I enzymes contain a tightly bound
74 FAD cofactor, are NADPH-dependent, possess two Rossmann folds for dinucleotide binding and a
75 conserved Baeyer-Villiger motif, a fingerprint sequence (FXGXXXHXXXW(P/D)) involved in catalysis
76 (22). Type II enzymes do not present the Baeyer-Villiger sequence motif, utilize FMN as coenzyme and
77 NADH as co-substrate whereas type 0 use FAD and NAD(P)H and lack the BVMO fingerprint motif
78 (23). A heme-containing BVMO has also been reported belonging to the cytochrome P450 superfamily
79 (24). Earlier studies had already suggested Baeyer-Villiger activity of other eukaryotic cytochrome P450
80 enzymes (25). This finding indicates that during evolution several different enzymes have evolved into
81 Baeyer-Villiger monooxygenases. Most biochemical and biocatalytic studies have been performed with
82 Type I BVMOs (23). This is partly due to the fact that they represent relatively uncomplicated
83 monooxygenase systems. These monooxygenases are typically soluble and composed of only one
84 component. Expression systems have been developed for a number of Type I BVMOs, while no
85 recombinant expression has been reported for Type II. All these enzymes are known to perform various
86 catalytic activities on different compounds even partially sharing their substrate profile. BVMOs are also
87 known to perform oxygenation on heteroatom-containing compounds. The first evidence of soft
88 nucleophilic containing substrate oxidation was reported with CHMO from *Acinetobacter* sp. that showed
89 4-tolyl ethyl sulfide conversion to the corresponding (S)-sulfoxide with a modest enantioselectivity (26).
90 BVMOs may also have medical relevance as in the case of Ethionamide monooxygenase (EtaA), a Type I
91 BVMO capable of converting a range of ketones into the corresponding esters (27). Except for catalyzing

92 Baeyer-Villiger oxidations, the enzyme is also able to oxidize the sulfide moieties of several
93 antitubercular thioamide drugs (27-28). The oxidized drugs appear to be highly toxic to the mycobacteria,
94 indicating that Ethionamide monooxygenase acts as a prodrug activator.

95 Phylogenetic analysis placed the sequence of Ar-BVMO together with *Mycobacterium tuberculosis* EtaA
96 (20) with only a distant relation to the other known class I BVMO proteins. *In vitro* experiments carried
97 out with the purified enzyme confirmed that this novel BVMO is indeed capable of typical Baeyer-
98 Villiger reactions as well as oxidation of the prodrug Ethionamide (20). Ortiz de Monellano's group (28)
99 demonstrated that Ethionamide is also oxidized by human flavin-containing monooxygenase 3 (FMO3).
100 Human FMO enzymes are a superfamily of flavoprotein monooxygenases involved in the detoxification
101 of a wide range of xenobiotics containing a soft nucleophile, usually nitrogen or sulfur (29). Since Ar-
102 BVMO has been shown to oxidize Ethionamide, we wondered whether this enzyme could play a role in
103 detoxification of xenobiotics, recognizing synthetic compounds such as drugs, typically recognized by
104 human FMO3. We took into consideration two synthetic drugs, Danusertib and Tozasertib, shown to be
105 N-oxygenated by human FMO3 (30-31) and certainly not present in the natural environment of this
106 bacterium. Species belonging to *Acinetobacter* genus have been reported as one of the major super-bugs,
107 resistant to antibiotic treatment (32). The presence in their genomes of genes coding for monooxygenases
108 similar to those involved in drug metabolism in humans becomes highly relevant when tackling the threat
109 they pose.

110 We report here that the overexpression of Ar-BVMO in *E. coli* renders it resistant to Imipenem and show
111 that this enzyme whilst mediating its usual Baeyer-Villiger oxidation on the carbonyl moiety of the β -
112 lactam ring, is also operating by an unprecedented mechanism of carbapenem resistance. In future, if
113 under physiological conditions appreciable carbapenemase activity is attributed to this monooxygenase, it
114 could represent a new target for drug design in the battle against carbapenems' resistance in
115 *Acinetobacter*.

116 MATERIALS and METHODS

117 Bacterial strains

118 *Escherichia coli* strain BL21 (DE3) (Invitrogen) transformed with the expression vector plasmid pT7
119 harbouring the gene coding for *Acinetobacter radioresistens* S13 Baeyer-Villiger monooxygenase (Ar-
120 BVMO) (20) and not transformed BL21 cells.

121 Reagents

122 FAD, acetonitrile, NADPH, Imipenem, methanol and salts were purchased from Sigma-Aldrich (Milan,
123 Italy). Tozasertib and Danusertib were purchased from Aurogene (Rome, Italy).

124 Sequence retrieval, alignment and phylogenetic analysis

125 Amino acid sequences from completely sequenced genomes of proteobacteria were retrieved from
126 GenBank database (<http://www.ncbi.nlm.nih.gov>). BLAST (33) probing of database was performed with
127 the BLASTP option of this program using default parameters. The ClustalW program (34) was used to
128 perform pairwise and multiple amino acid sequence alignments.

129 Preliminary multiple alignments of amino acid sequences were generated with the program ClustalW
130 using default gap penalties. The selection of characters eligible for the construction of phylogenetic tree
131 was optimized by comparing entire sections of all the available BVMO alignments with comprehensive
132 inventories of significant binary alignments obtained by BLAST probing of the protein database with
133 representative bacterial sequences. The structure and organization of the BVMOs were deduced from the
134 data present in GenBank from the microorganisms available in data banks. BLAST probing of the protein
135 databases was performed with the BLASTP program choosing sequences with an E-value of 0.0, 100%
136 query coverage and a threshold of 99% sequence identity. For construction of the phylogenetic tree, a
137 representative of each class of BVMO was selected to demonstrate its relationship to the Ar-BVMO.

138 The phylogenetic tree was constructed using the Distance Matrix (DM) method using MEGA version 4
139 (35), after multiple sequence alignment and truncation to the same length. Distances according to the
140 Kimura two-parameter model (36) and clustering with the neighbor-joining method (37) were determined
141 using bootstrap values based on 1,000 replications.

142 **Expression and purification of recombinant Ar-BVMO**

143 The expression and purification of Ar-BVMO were performed as previously reported (20). After Ni-NTA
144 affinity chromatography, the heterologous expression of Ar-BVMO yielded 8 mg of purified protein per
145 litre of culture. The concentration of the pure Ar-BVMO was determined by spectroscopy with the peak
146 absorbance at 450 nm and the extinction coefficient of free FAD ($\epsilon_{450} = 11,300 \text{ M}^{-1} \text{ cm}^{-1}$).

147 **Ar-BVMO incubation with Tozasertib, and Danusertib**

148 N-oxygenation of the anticancer drugs Danusertib and Tozasertib was evaluated by incubating a mixture
149 of 0.3 μM of purified enzyme in 50 mM potassium phosphate buffer (pH 7.4), 0.5 mM NADPH and
150 increasing amounts of substrates (6, 12.5, 25, 50, 75, 100, 150, 200, 300 and 400 μM) in a final volume of
151 200 μl . Incubations were carried out at 37°C for 15 min and were terminated by the addition of 100 μl of
152 ice-cold methanol. The aqueous supernatant was centrifuged at 2,000x g for 10 min and was subjected to
153 High Pressure Liquid Chromatography. Kinetic parameters related to the turnover were determined
154 through Michaelis-Menten kinetics using SigmaPlot Software.

155 **Analysis by High Pressure Liquid Chromatography (HPLC)**

156 HPLC was performed on an Agilent quaternary pump HPLC system (Agilent Technologies) equipped
157 with an analytical C18 column (4.6 x150 mm, 5 μm). The method to separate Tozasertib and “Tozasertib
158 N-oxide” and Danusertib from “Danusertib N-oxide” used a mobile phase of 78% methanol and 22%
159 distilled water at a flow rate of 1.0 ml/min. The formation of Tozasertib N-oxide was monitored at the
160 wavelength of 250 nm while the Danusertib N-oxide product was monitored at 295 nm (30-31). The

161 kinetic analysis of N- or S-oxygenation was carried out using a nonlinear regression analysis program
162 (SigmaPlot).

163 **Susceptibility testing**

164 Imipenem sensitivity of *E. coli* BL21 cells (i) transformed with pT7-Ar-BVMO induced with IPTG, (ii)
165 not induced and, (iii) negative control- transformed with pT7 (carrying another gene (38)) induced with
166 IPTG, was performed by disc diffusion method (39) according to the latest updated guidelines (2014) of
167 the European Committee of Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org) using
168 standard antibiotic disks (Oxoid) and Mueller-Hinton agar (MHA) plates (Sigma). There were five
169 replicates for each condition tested. Inhibition zones measured as mean diameter were categorized as
170 'susceptible' (S), 'intermediary' (I) and 'resistant' (R) according to EUCAST standards.

171 The effect of Ar-BVMO activity on the antimicrobial properties of Imipenem was subsequently assessed
172 by a microbiological assay. Inactivation reaction contained 3 mM Imipenem, 2 mM NADPH and 100 μ M
173 of purified Ar-BVMO in 50 mM phosphate buffer pH 7.4, in a total volume of 0.5 ml. The reaction was
174 allowed to proceed for 30 min at 37°C. A control reaction was performed in the same conditions
175 described above without adding NADPH. Fifty μ l aliquots of the two reactions were used for agar
176 diffusion plate tests (40) in order to evaluate the effect of metabolized Imipenem on the growth of
177 sensitive *E. coli* BL21 cells. MH agar plates were prepared using sterile MHA. Upon solidification of the
178 agar medium, sensitive not transformed BL21 and transformed not induced BL21 cells were spread over
179 the plate surface at a concentration of 0.5 MacFarland. Then, a well was made in the centre of each plate
180 using a sterile Pasteur pipette. After adding 50 μ l of the Ar-BVMO-Imipenem reaction with and without
181 NADPH, an uninoculated agar plug larger than the well was placed over it to prevent volatilization of the
182 enzymatic reaction into the headspace of the Petri dish (40). The diameter of the growth inhibition zone
183 was measured after 24 hours from the application of the test substance at 37°C. There were five replicates
184 for each condition tested.

185 **Enzymatic activity assay**

186 Enzyme activity was measured spectrophotometrically by monitoring the substrate-dependent decrease in
187 NADPH concentration at 340 nm ($\epsilon_{340 \text{ nm}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). Reaction mixtures contained 50 mM Tris-
188 HCl pH 8.0, 200 μM NADPH, and a range from 5 to 150 μM of Imipenem using air-saturated buffers.
189 Steady state kinetic parameters were determined by fitting the initial rates, calculated from the first 200 s
190 of the reaction, to the standard Michaelis-Menten equation using SigmaPlot program. Experiments were
191 conducted in triplicates.

192 **LC-MS/MS analysis**

193 The chromatographic separations were run on a Phenomenex Luna C18 column, $150 \times 2.0 \text{ mm}$, 3 μm
194 particle size (Phenomenex, Torrance, CA, USA). Injection volume was 10 μL and flow rate 0.2 mL min^{-1} .
195 Isocratic mobile phase composition was adopted: 5:95 acetonitrile/aqueous formic acid 0.05%. A LTQ -
196 Orbitrap hybrid mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an atmospheric
197 pressure interface and an ESI ion source was used. The LC column effluent was delivered into the ion
198 source using nitrogen as sheath and auxiliary gas. The needle voltage was set at the 4.5 kV value. The
199 heated capillary value was maintained at 270°C . The acquisition method used was previously optimized
200 in the tuning sections for the analyte ion (capillary, magnetic lenses and collimating octapoles voltages) in
201 order to achieve the maximum of sensitivity. Spectra were acquired in the positive HRMS and MS/MS
202 mode, precursor ions 300 and 316, normalized collision energy 25 %, mass range 320-80 m/z .

203 **RESULTS**

204 Phylogenetic tree of the Ar-BVMO was inferred from the selected sequences by DM method as
205 mentioned in Materials and Methods section. As shown in Figure 1, the clade comprising *Mycobacterium*
206 *tuberculosis* EtaA, Ar-BVMO and *A. baumannii* EtaA belong to a separate group, distant from the other
207 BVMO proteins. Furthermore, Ar-BVMO shares 100% amino acid sequence identity with the

208 Ethionamide monooxygenase of MDR *A. baumannii* strains (GenBank ID: gb|EXB35767.1|,
209 gb|EXB73644.1|, gb|EXE14728.1| and gb|KCX39290.1|).

210 Previously in our laboratory we had tested different therapeutic drugs as possible substrates of this human
211 hepatic enzyme including Danusertib and Tozasertib (29-30). Tozasertib (VX-680) and Danusertib (PHA-
212 739358) are inhibitors of Aurora kinases which have been identified as a potential target in anticancer
213 therapy. As a starting point, the possibility of these two inhibitors acting as substrates for Ar-BVMO
214 enzyme was tested by investigating whether any enzymatic products could be detected. After each set of
215 reactions at 37 °C in the presence of NADPH, the product(s) was separated by HPLC and the data
216 obtained are shown in Figure 2. As can be seen from in the figure, the expected N-oxides were detected
217 with shorter retention times of 3.7 min for “Danusertib N-oxide” (Figure 2A) and 4.1 min for “Tozasertib
218 N-oxide” (Figure 2C), in agreement with the previously published data by our group (30-31). The
219 catalytic activity of Ar-BVMO towards two kinase inhibitors, Danusertib and Tozasertib, was
220 investigated. Enzymatic reactions were carried out with the purified recombinant Ar-BVMO in the
221 presence of NADPH and increasing amounts of each drug as reported in the experimental section. Kinetic
222 parameters for the N-oxygenation for Danusertib (Figure 2B) and Tozasertib (Figure 2D) were determined
223 by nonlinear regression analysis with a calculated K_M value of $352 \pm 58 \mu\text{M}$ and $54.3 \pm 8.3 \mu\text{M}$,
224 respectively. The corresponding V_{\max} values of 8.1 and 7.4 nmol/min/mg of protein were also calculated
225 for Danusertib and Tozasertib, respectively. These results confirm that the bacterial enzyme is indeed able
226 to metabolize these two synthetic drugs.

227 ***Acquisition of Imipenem resistance by Escherichia coli overexpressing Ar-BVMO-*** Once it was
228 proven that the bacterial Ar-BVMO is capable of metabolizing the synthetic drugs tested, our attention
229 was turned to antibiotics as another class of synthetic drugs and directly relevant to the MDR effects seen
230 in *Acinetobacter* spp. Initially, in order to understand whether the expression of Ar-BVMO in Imipenem
231 sensitive *E. coli* BL21 cells could confer resistance to Imipenem (i.e. enzymatic reaction of Ar-BVMO
232 with Imipenem resulting in a product no longer active as an antibiotic), a disc diffusion assay was
233 performed following EUCAST guidelines (www.eucast.org). According to EUCAST clinical breakpoint

234 table v. 5.0 (valid from 2015/01/01) for *Enterobacteriaceae*, the zone diameter breakpoint for Imipenem
235 is ≥ 22 mm for sensitivity and < 16 mm for resistance, corresponding to MIC values of 2 and 8 mg/L of
236 Imipenem, respectively, for sensitive and resistant *Enterobacteriaceae* including *E. coli*, which is the most
237 important model organism of this family. As shown in Figure 3, the average diameter of the inhibition
238 zone for *E. coli* BL21 cells (Figure 3 top panel-A), the same *E. coli* strain transformed with pT7-Ar-
239 BVMO but not induced (Figure 3 top panel-B) and *E. coli* strain transformed with pT7 (negative control)
240 IPTG-induced (Figure 3 top panel-C) was measured as 25.5 mm, 24.0 mm, and 28.0 mm, respectively.
241 The results clearly show that the *E. coli* BL21 cells are sensitive to Imipenem (zone diameter breakpoint
242 ≥ 22 mm) but there is no visible inhibition zone when *E. coli* BL21 cells are transformed and the
243 expression of Ar-BVMO is induced by IPTG i.e. the cells became resistant to Imipenem (zone diameter
244 breakpoint < 16 mm), Figure 3 top panel-D. These data provided the preliminary but encouraging
245 evidence that the over expression of Ar-BVMO in Imipenem sensitive cells may result in antibiotic
246 resistance.

247 ***Ar-BVMO catalyses the inactivation of Imipenem-*** The effect of Ar-BVMO activity on the
248 antimicrobial properties of Imipenem was further studied by a microbiological assay (41). The reaction
249 product(s) of purified Ar-BVMO and Imipenem was loaded in a well in the MH agar plates where
250 Imipenem sensitive BL21 cells and BL21 carrying pT7-Ar-BVMO whose gene expression was not
251 induced, were grown at 37°C for 24 h. As shown in Figure 3 bottom panel-B and D Imipenem is not
252 active after the reaction with Ar-BVMO since both not transformed and transformed but not induced
253 BL21 exhibited an inhibition zone of 12.0 mm and 0.5 mm, respectively. When the enzymatic reaction
254 without NADPH (negative control) was deposited in the agar well the diameter of the inhibition zone
255 were 24.0 mm (Figure 3 bottom panel-A) and 24.5 mm (Figure 3 bottom panel-C) for not transformed
256 BL21 and transformed but not induced BL21, respectively. These data also support the idea that the
257 enzymatic reaction of Ar-BVMO converts Imipenem into an inactive compound no longer possessing its
258 original antibacterial property.

259 *Catalytic activity of purified Ar-BVMO in presence of Imipenem and identification of the*
260 *products-* Subsequent to the *in vivo* data, a spectroscopic method was used to measure the activity of the
261 purified Ar-BVMO in the presence of Imipenem. In order to determine the steady state kinetic parameters
262 for Ar-BVMO turnover, the NADPH consumption assay which has previously been described in literature
263 as an efficient system to evaluate K_M (42), was used. This method relies on the spectrophotometric
264 monitoring of the consumption of the co-substrate NADPH at 340 nm, in the presence of increasing
265 concentrations of Imipenem. The experimental data from the NADPH consumption were fitted to a
266 Michaelis-Menten curve used for the determination of a K_M value of $12.3 \pm 0.8 \mu\text{M}$ (Figure 4) and a V_{max}
267 value of $468.2 \pm 9.6 \text{ nmol/min/mg}$ of protein. These values are in the same range as those reported for
268 known substrates of other BVMO enzymes (42) indicating that Imipenem could be a real substrate of Ar-
269 BVMO.

270 In order to identify the product(s) of the Ar-BVMO reaction with Imipenem, LC-MS experiments were
271 carried out. Imipenem incubation ($100 \mu\text{M}$) was performed in phosphate buffer (50 mM , $\text{pH } 7.4$) at room
272 temperature and monitored by LC-high resolution mass spectrometry at different reaction times. Ar-
273 BVMO and NADPH concentrations were 0.45 and $200 \mu\text{M}$, respectively. The incubation mixture was
274 simply ten-fold diluted using elution mobile phase and injected in the LC-MS instrument. Imipenem peak
275 was eluted at 4.7 min . The main metabolite was detected as a double peak (5.0 and 5.5 min) due to a
276 mixture of isobaric products. The accuracy of orbitrap high resolution mass determination allows to
277 deduce that the elemental composition of the two compounds correspond to $\text{C}_{12}\text{H}_{18}\text{N}_3\text{O}_5\text{S}$ with 1.05 ppm
278 of error (MH^+ ion at 316.0962 m/z). A classical Baeyer-Villiger asymmetric oxygen insertion can explain
279 this finding as shown in Figure 5.

280 An analogous BV catalytical synthetic reaction was described regarding a similar lactam
281 (bicyclo[3.2.0]hept-3-en-1-one) (43). In order to elucidate the structure, a MS/MS study was performed.
282 High resolution MS/MS spectra of Imipenem and the corresponding Ar-BVMO oxidized products are
283 shown in figures 6a and 6b, respectively. The MS^n study of Imipenem precursor ion (300.0867 m/z)

284 allows to hypothesize the fragmentation pathway shown in Figure 7. The main fragmentation pathways
285 are the (consecutive) losses of water and carbon dioxide. The elimination of the amidino-ethylthio- lateral
286 chain is noteworthy. High resolution mass defect data were fundamental to differentiate CO₂ and NH-CH-
287 NH₂ losses, both with nominal mass of 44 Da but with different significant digits. The interpretation of
288 MS/MS spectrum of Ar-BVMO metabolites is reported in Figure 8. A double CO₂ loss characterizes the
289 spectrum. The intense ion due to the loss of the amidino-ethylthio lateral chain confirms that oxygen
290 insertion occurred on the lactamic ring. A possible intramolecular hydrogen bond justifies both the
291 proposed formation of a new lactone cyclic structure and the chromatographic behavior of the two
292 metabolites which are more retained than the parent compound on the reverse phase column. Successive
293 losses of water, ammonia and ethylamidine molecules could explain all the obtained fragment ions. The
294 same fragmentation pathway is valid in the interpretation of MS/MS spectrum of the ion corresponding to
295 the asymmetrical isomer reported in Figure 5.

296 DISCUSSION

297 The World Health Organization (WHO) has recently classified antibiotic resistance as one of the three
298 greatest threats to human health. The β -lactams are one of the most important and frequently used
299 classes of antibiotics in medicine and are essential in the treatment of serious gram-negative infections.
300 The past two decades have seen substantial increases in the utilization of carbapenems such as Imipenem
301 and Meropenem. Predictably, this increase in carbapenem consumption has been accompanied by the
302 emergence of carbapenem-resistant gram-negative pathogens (44). The initial and seemingly unstoppable
303 success of antibiotics has been countered by an escalation of resistance mechanisms in bacteria with the
304 emergence of many genera of bacteria that are resistant to all antibiotics (45-47). The genus *Acinetobacter*
305 epitomizes this trend and deserves close attention. *Acinetobacter* spp. display mechanisms of resistance to
306 all existing antibiotic classes as well as a prodigious capacity to acquire new determinants of resistance
307 (3, 48-49).

308 Furthermore, *Acinetobacter* spp. appears to be well suited for genetic exchange and is among a unique
309 class of gram-negative bacteria that are described as “naturally transformable” (50). The increasing
310 recovery in the clinic of multidrug-resistant *A. baumannii* is a frightening reality (6). A previously
311 published study describing the genome sequences of both susceptible (SDF) and resistant (AYE) isolates
312 of *A. baumannii* has shed light on the abundance of resistance genes found in this organism (51). Fournier
313 and co-workers identified a resistance island in AYE that contained a cluster of 45 resistance genes in the
314 MDR isolate. Among the key resistance genes identified were those coding for β -lactamases, various
315 aminoglycoside-modifying enzymes, and tetracycline efflux pumps. A significant fraction of the open
316 reading frames were located in putative alien islands, indicating that the genome acquired a large amount
317 of foreign DNA. More than 30 years ago it was demonstrated that *Acinetobacter* spp. could acquire
318 antimicrobial resistance factors through conjugation of plasmids (52). The genome of *A. baumannii* not
319 only contains a gene coding for an Ethionamide monooxygenase that phylogenetically is part of the same
320 group as that of Ar-BVMO but also shares a 100% amino acid sequence identity with Ar-BVMO. As
321 mentioned earlier, human FMO3, Ar-BVMO and *M. tuberculosis* EtaA can metabolize a common
322 substrate, ethioamide. Based on this, we investigated the ability of Ar-BVMO to use as substrate two
323 potent and selective Aurora kinase inhibitors, Danusertib and Tozasertib, used for the treatment of solid
324 tumors and hematopoietic cancers and metabolized by human FMO3 in liver microsomes (53-54).

325 *In vitro* experiments with recombinant Ar-BVMO demonstrated that both Danusertib and Tozasertib are
326 monooxygenated by Ar-BVMO with K_M values comparable to those obtained with recombinant human
327 FMO3 (30). The reason why a prokaryotic flavin monooxygenase should metabolize substrates that are
328 typically metabolized by the eukaryotic FMO enzymes is not clear. Since both proteins, together with
329 EtaA belong to the same class of flavoproteins and are involved in detoxification processes, it can be
330 speculated that during evolution some FMO proteins became BVMOs providing the acquiring organism
331 new metabolic capabilities. This is not the first example of a bacterial FMO that shares the same substrate
332 with a human flavoprotein. Chen and co-workers (55) showed that a FMO from *Methylocella silvestris* is

333 able to oxidize trimethylamine that is a typical substrate of eukaryotic FMO enzymes. It can be assumed
334 that the *A. baumannii* enzyme would be able to monooxygenate the two anticancer drugs even if further
335 experiments are required to confirm this. It could be hypothesized that genetic exchange between the
336 progenitor (*A. radioresistens*) and its recipient of clinical relevance (*A. baumannii*), may have occurred in
337 humans (19) leading to the BVMO acquisition by the latter species. Both *A. radioresistens* and *A.*
338 *baumannii* are identified on the human skin, especially in hospitalized patients, it is therefore possible that
339 BVMO gene exchange may have occurred at this location (4). In order to provide support for the
340 hypothesis of lateral gene transfer, the genetic context of the Ar-BVMO (Accession number GG705134.1)
341 was evaluated and compared to that of EtaA of *Acinetobacter baumannii* strains 1461402 (Accession
342 number JEW01000001) 230853 (Accession number JEW01000002), 983759 (Accession number,
343 JEX001000002); 263903-1 (Accession number JMN01000001). The genes flanking upstream and
344 downstream of the Ar-BVMO were found to be not only identical but also in the same position as those of
345 the four *A. baumannii* strains. In addition, the GC content (56) of the ethionamide monooxygenase genes
346 of the four *A. baumannii* strains was compared with *A. radioresistens* genome content. The GC% was
347 calculated using the Endmemo software (www.endmemo.com/bio/gc.php) and the values were 42.65%
348 and 42.69% for *A. baumannii* Eta gene and Ar-BVMO of *A. radioresistens*, respectively. These data are
349 not in support of a horizontal gene transfer unless a large portion of the genome has been transferred from
350 *A. radioresistens* to *A. baumannii* and therefore a more in depth analysis is required which is outside the
351 scope of this work.

352 The presence of a flavin monooxygenase in a MDR bacterium that has 100% sequence identity with Ar-
353 BVMO (i.e. identical protein) with pro-drugs as substrates strongly suggests that the EtaA could be used
354 by *A. baumannii* to metabolize/detoxify different types of synthetic drugs including antibiotics in exactly
355 the same way as Ar-BVMO.

356 In this work we have demonstrated that sensitive *E. coli* cells overexpressing Ar-BVMO became resistant
357 to the antibiotic Imipenem. NADPH consumption assay shows that Imipenem is a substrate of Ar-BVMO

358 and agar well diffusion assay proves that the enzyme destroys the antibacterial property of the antibiotic.
359 Like all other β -lactams, Imipenem inhibits bacterial cell wall synthesis by binding to and inactivating
360 penicillin binding proteins. Antibiotic resistance can occur via three general mechanisms: prevention of
361 interaction of the drugs with target, efflux of the antibiotic from the cell, and direct destruction or
362 modification of the compound. The most prevalent resistance mechanism against β -lactams is the
363 production of β -lactamases. Among the β -lactamases, metalloenzymes are distinguished by having a
364 zinc ion required for enzymatic activity (57). Sometimes referred to as carbapenemases, they are able to
365 hydrolyze many β -lactam antibiotics including carbapenems (58). In the present work, for the first time,
366 we demonstrate that a bacterial flavin monooxygenase is able to metabolize Imipenem in a way that it is
367 no longer active by modification of the parent compound. Two molecules with evidence of the
368 transformation of the lactam ring, according to Baeyer-Villiger oxygen insertion mechanism, were
369 identified by LC-HRMS analysis. This is not the first report of a flavin monooxygenase catalysing
370 antibiotic inactivation since Yang and co-workers have previously reported TetX, a flavin-dependent
371 monooxygenase from bacteria of the genus *Bacteroides*, that regioselectively hydroxylates the
372 tetracycline substrate (41). However, they could not capture the product and suggested that the resulting
373 hydroxylated product was an unstable compound that underwent a non-enzymatic decomposition (41).

374 Future efforts will be focused on the screening of other carbapenem members and, if under physiological
375 conditions appreciable carbapenemase activity is attributed to this monooxygenase enzyme, it could
376 represent a new target for drug design in the battle against carbapenems' resistance in *Acinetobacter*.

377

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380

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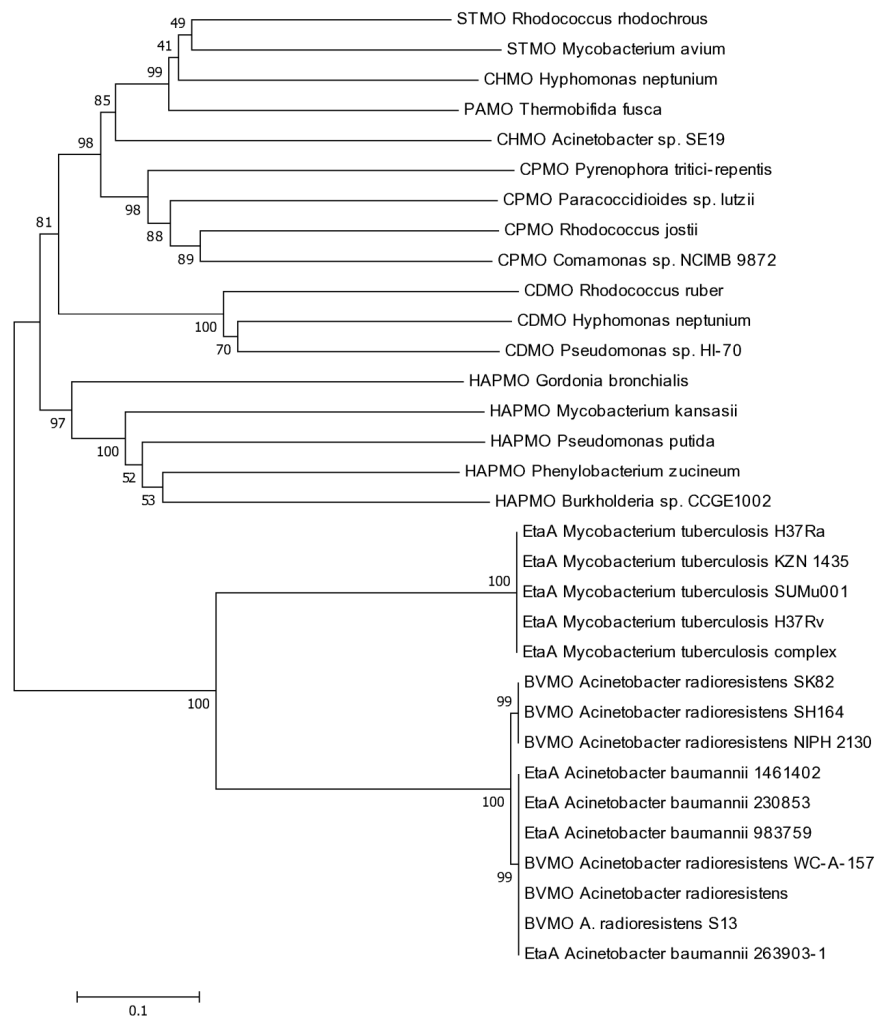


FIGURE 1. Phylogenetic position of Ar-BVMO in relation to the other BVMOs and *Acinetobacter baumannii* Ethionamide monooxygenase. Neighbor-joining consensus tree.

STMO *Rhodococcus rhodochrous*= steroid monooxygenase (BAA24454.1); STMO *Mycobacterium avium*= steroid monooxygenase (BAN31365.1); CHMO *Hyphomonas neptunium*= steroid monooxygenase (YP_760152.1); PAMO *Thermobifida fusca*= phenylacetone monooxygenase (Q47PU3.1); CHMO *Acinetobacter* sp. SE19= cyclohexanone monooxygenase (AF282240_5); CPMO *Pyrenophora treitici-repentis*= cyclopentanone monooxygenase (XP_001942142.1); CPMO *Paracoccidioides* sp. *lutzi*= cyclopentanone monooxygenase (XP_002792362.1); CPMO *Rhodococcus josti*= cyclopentanone monooxygenase (YP_703208.1); CPMO *Comamonas* sp. NCIMB 9872= cyclopentanone monooxygenase (BAC22652.1); CDMO *Rhodococcus ruber*= cyclododecanone monooxygenase (AAL14233.1); CDMO; *Hyphomonas neptunium* ATCC33707= cyclododecanone monooxygenase (ABI76430.1); CPDMO *Pseudomonas* sp. HI-70= cyclopentadecanone monooxygenase (BAE93346.1); HAPMO *Gordonia bronchialis*= hydroxyacetophenone monooxygenase (ACY20506.1); HAPMO *Mycobacterium kansasii*= hydroxyacetophenone monooxygenase (ZP_04749205.1); HAPMO *Pseudomonas putida*= hydroxyacetophenone monooxygenase (ACJ37423.1); HAPMO *Phenylobacterium zucineum*= hydroxyacetophenone monooxygenase (YP_002130647.1); HAPMO *Burkholderia* sp. CCGE1002= hydroxyacetophenone monooxygenase (ADG19710.1); EtaA *Mycobacterium tuberculosis* H37Ra= Ethionamide monooxygenase (YP_001285245.1); EtaA *Mycobacterium tuberculosis* KZN1435= Ethionamide monooxygenase (YP_003033907); EtaA *Mycobacterium tuberculosis* SUMu001= Ethionamide monooxygenase (ZP_07416557.1); EtaA *Mycobacterium tuberculosis* H37Rv= Ethionamide monooxygenase (NP_218371.1); EtaA *Mycobacterium* complex= Ethionamide monooxygenase (WP_003910007.1); BVMO *Acinetobacter radioresistens* SK82= Baeyer-Villiger monooxygenase (EET82318.1); BVMO *Acinetobacter radioresistens* SH164= Baeyer-Villiger monooxygenase (EEY85682.1); BVMO *Acinetobacter radioresistens* NIPH 2130= Baeyer-Villiger monooxygenase (ENV85773.1); EtaA *Acinetobacter baumannii* 1461402= Ethionamide monooxygenase (EXB35767.1); EtaA *Acinetobacter baumannii* 230853= Ethionamide monooxygenase (EXB73644.1); EtaA *Acinetobacter baumannii* 983759= Ethionamide monooxygenase (EXE14728.1); BVMO *Acinetobacter radioresistens* WC-A-157= Baeyer-Villiger monooxygenase (EJQ34398.1); BVMO *Acinetobacter radioresistens*= Baeyer-Villiger monooxygenase (ADF32068.2); BVMO *Acinetobacter radioresistens* S13= Baeyer-Villiger monooxygenase (GU145276.2); EtaA *Acinetobacter baumannii* 263903-1= Ethionamide monooxygenase (KCX39290.1).

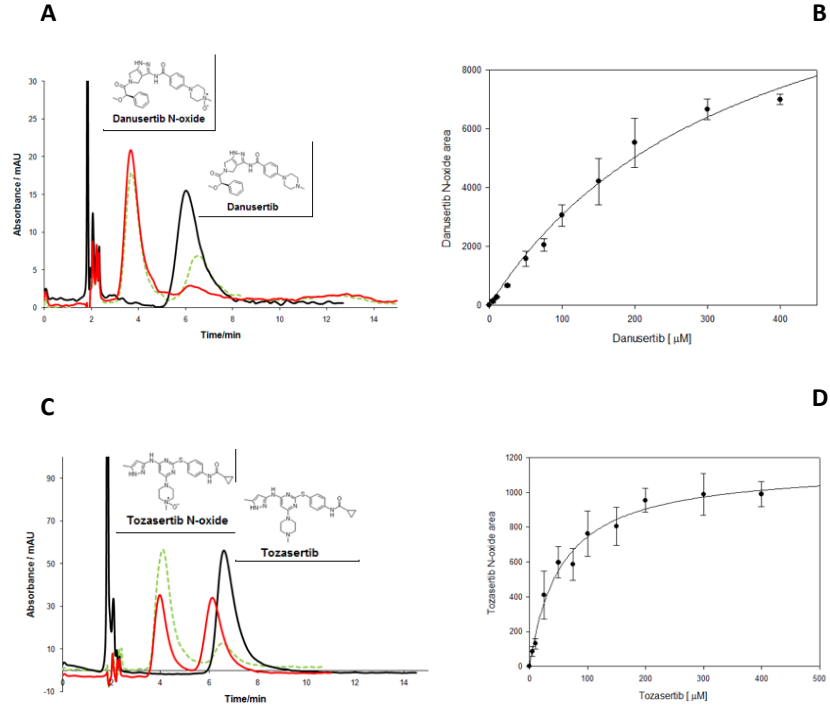


FIGURE 2. Danusertib and Tozasertib oxidation by purified recombinant Ar-BVMO. HPLC traces for Danusertib (**A**) and Tozasertib (**C**) conversion. The red trace shows Ar-BVMO reaction, the black trace shows the negative control without enzyme, the green trace shows the positive control with purified recombinant human FMO3. The retention times are 6.4 min for Danusertib and 3.9 min for the product “Danusertib N-oxide” and 6.5 min for Tozasertib and 4.4 min for the product “Tozasertib N-oxide”. Michaelis Menten curve of the enzymatic “Danusertib N-oxide” (**B**) and “Tozasertib N-oxide” (**D**) product formed in the presence of increasing amount of Danusertib and Tozasertib, respectively.

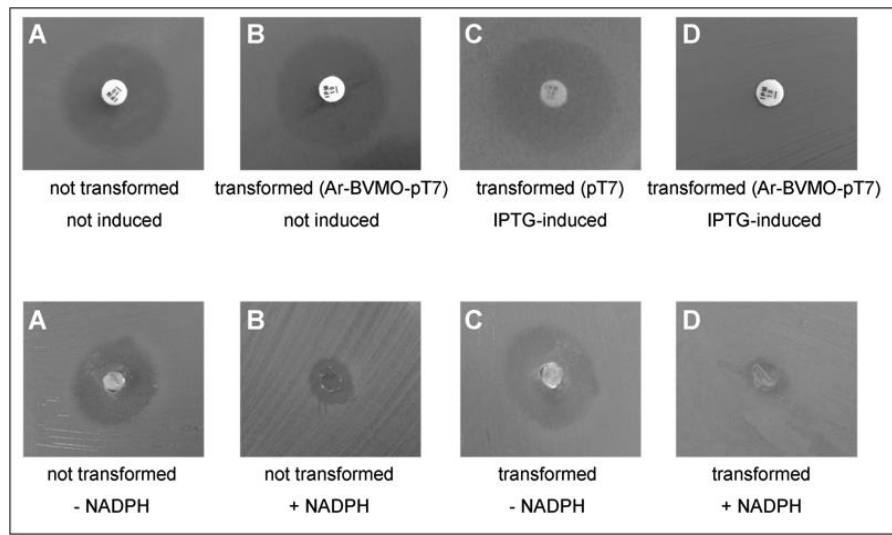


FIGURE 3. Imipenem antibiograms of *E. coli* BL21 cells against disk embedded Imipenem (Top panel). *E. coli* BL21 cells were plated on Muller Hinton agar plate at a concentration of 0.5 MacFarland and incubated overnight at 37°C. A) *E. coli* BL21; B) *E. coli* BL21 transformed with Ar-BVMO-pT7 but not induced; C) *E. coli* BL21 transformed with pT7 plasmid and induced with IPTG (negative control); D) *E. coli* BL21 transformed with pT7-Ar-BVMO and induced with IPTG.

Agar well diffusion assay (Bottom panel). Imipenem sensitive not transformed (A, B) and transformed but not induced BL21 cells (C, D) were spread over the plate surface of MHA at a concentration of 0.5 MacFarland. Fifty ml of the reaction product/s between Ar-BVMO and Imipenem were placed inside a well in the center of each plate. B, D: reaction with NADPH; A, C: reaction without NADPH.

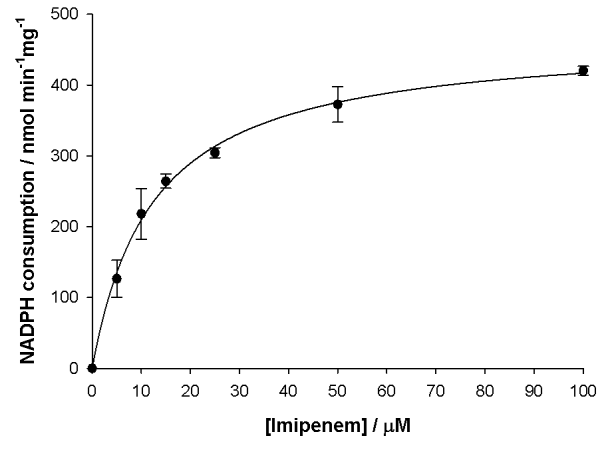


FIGURE 4. NADPH consumption assay for Ar-BVMO in the presence of Imipenem. Plot of enzymatic reaction velocity versus Imipenem concentration fitted to the Michaelis-Menten equation ($R^2 = 0.998$). Data are shown as mean \pm SD of three different measurements.

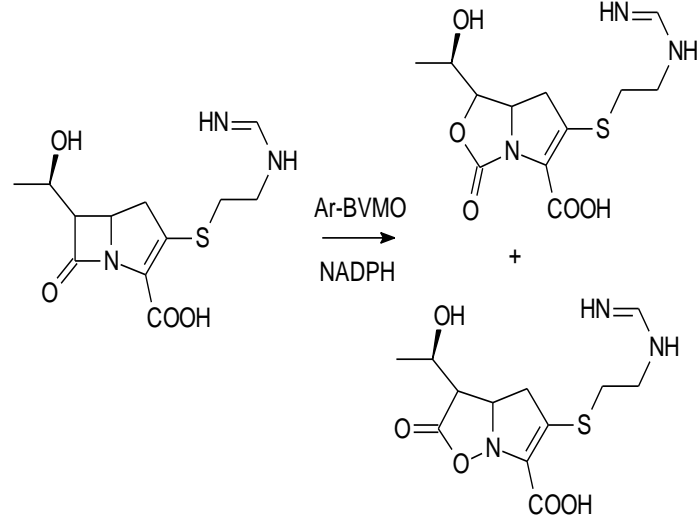


FIGURE 5. Asymmetric oxygen insertion in the carbapenem ring of imipenem according to the classical Baeyer-Villiger mechanism.

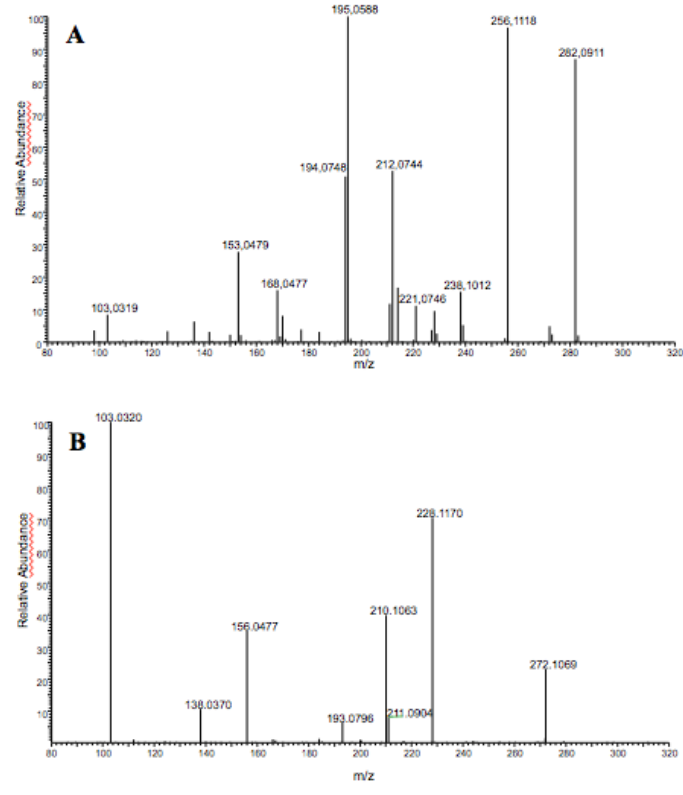


FIGURE 6. MS/MS high-resolution spectra of Imipenem and its metabolite. Spectrum of Imipenem 300.0987 m/z precursor ion (A) and spectrum of the first eluted M+O metabolite 316.0965 m/z (B) precursor ion acquired at 25% collision energy, in the range 80-320 m/z.

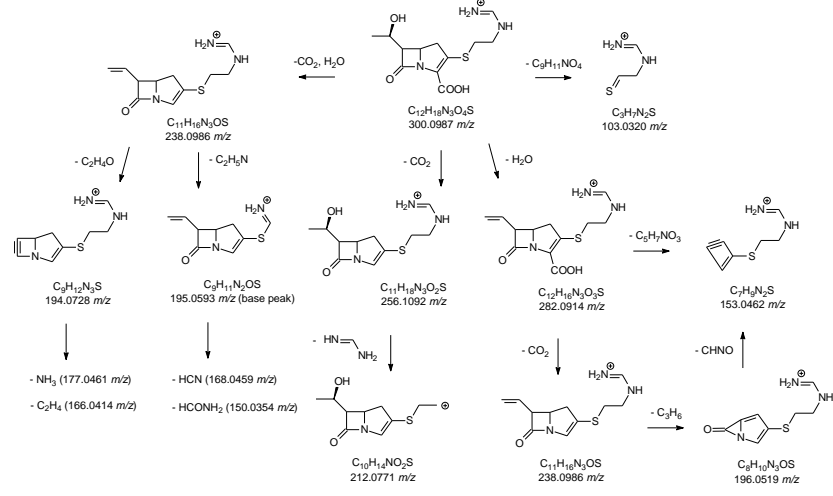


FIGURE 7. The hypothesized fragmentation pathway of Imipenem precursor ion (300.0867 m/z).

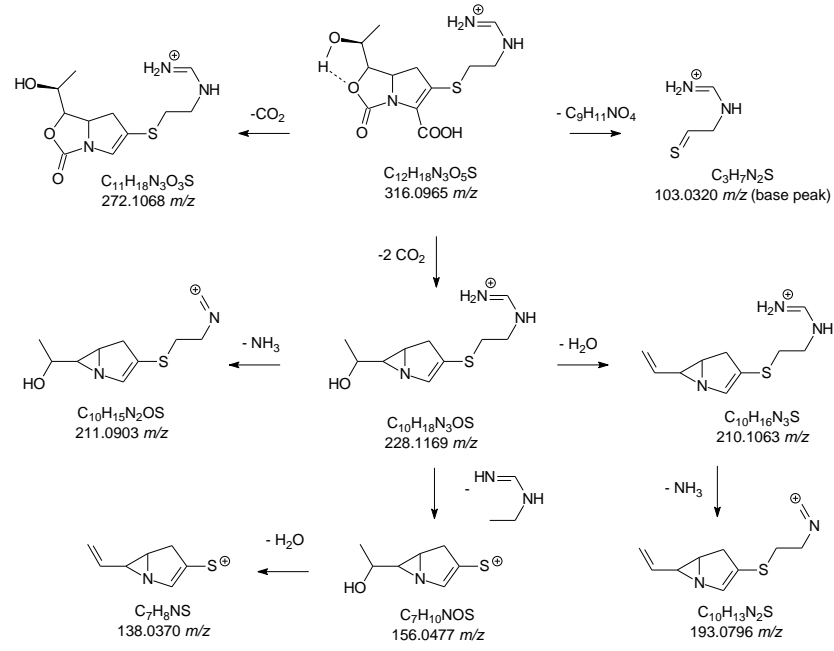


FIGURE 8. The interpretation of MS/MS spectrum of Ar-BVMO metabolite.